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(54) Title: USE OF MALATE DEHYDROGENASE FOR NADH REGENERATION

(57) Abstract: The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the present invention relates to an enzymatically operating process, in which, in a coupled enzymatic reaction system, NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second enzyme system. A reaction system which operates according to the invention in this manner and an advantageous whole cell catalyst are also proposed.

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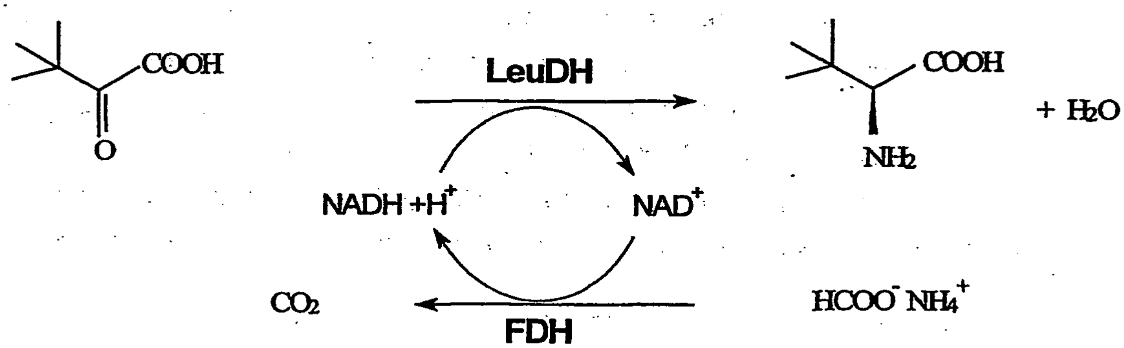
Use of malate dehydrogenase for NADH regeneration

The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the present invention relates to an enzymatically operating process, in which, in a coupled enzymatic reaction system, NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second enzyme system.
A reaction system which operates according to the invention in this manner and an advantageous whole cell catalyst or suitable plasmids are also proposed.

The production of optically active organic compounds, e.g. alcohols and amino acids, by a biocatalytic route is increasingly gaining importance. The coupled use of two dehydrogenases with cofactor regeneration has emerged, inter alia, as a route for the large-scale industrial synthesis of these compounds, in particular alcohols and amino acids (DE19753350, EP118750).

Equation 1:



In situ regeneration of NADH with the NAD-dependent formate dehydrogenase from *Candida boidinii* in the reductive amination of trimethyl pyruvate to give L-tert-leucine (Bommarius et al. Tetrahedron Asymmetry 1995, 6, 2851-2888).

In addition to their catalytic property and efficiency, the biocatalysts efficiently employed in an aqueous medium furthermore have the advantage that in contrast to a large number of synthetic metal-containing catalysts, the use of metal-containing starting substances, in particular those which contain heavy metals and are therefore toxic, can be dispensed with. The use of expensive and furthermore hazardous reducing agents, such as, for example, borane, in the case of asymmetric reduction can also be dispensed with.

The FDH, e.g. from *Candida boidinii*, successfully employed to date in these systems has the disadvantage that the specific activity of this enzyme class at 4-8 U/mg is very low. This necessitates the use of a large amount of expensive enzyme with recycling which is difficult from the apparatus point of view if a process designed in this way is to be carried out advantageously under economic aspects on an industrial scale.

Malate dehydrogenase (MDH), called "malic enzyme" catalyses the oxidative decarboxylation of malate to pyruvate. Numerous malate dehydrogenases from various organisms are known, thus, inter alia, from higher animals, plants and microorganisms. A distinction is made between four types of malate dehydrogenases, which are classified into the enzyme classes E.C. 1.1.1.37 to E.C. 1.1.1.40 (http://www.genome.ad.jp). NAD and/or NADP is required as a cofactor, depending on the type of malate dehydrogenase.

On the basis of the irreversibility of the oxidative decarboxylation reaction of L-malic acid to pyruvate, the use of malate dehydrogenase is also appropriate in the systems described above in respect of a favourable cofactor regeneration.
The use of malate dehydrogenase for regeneration of NAD is described, for example, by Suye et al. in a work from 1992 (S.-I. Suye, M. Kawagoe, S. Inuta, *Can. J. Chem. Eng.* **1992**,

70, 306-312). NADH regeneration by means of malate dehydrogenase is used here for reductive amination of pyruvate by means of an alanine dehydrogenase, NADH being consumed. The pyruvate is formed by oxidative decarboxylation from L-malic acid, and is immediately consumed again in the following step by alanine dehydrogenase. A concentration of pyruvate in the reaction solution is therefore avoided, whereby the problem of any inhibitions of the enzymes involved by the presence of amounts of pyruvate in the stoichiometric range is also eliminated. Nevertheless, the system described is limited exclusively to the production of alanine (see also S.-I. Suye, *Recent Res. Devel. Ferment. Bioeng*. **1998**, *1*, 55-64).

Interestingly - in spite of the technically high potential - apart from these works by the Suye study group no further works using "malate dehydrogenase" for regeneration of the oxidized cofactor NAD+ during reductions of ketones or reductive aminations of keto acids have been described.

The object of the present invention was therefore to provide a further process for the preparation of chiral organic compounds, such as amino acids or alcohols, which can be obtained by a coupled enzymatic reaction system as described above, using malate dehydrogenase, which is not limited to the preparation of one substance. In particular, it should be possible to employ this process on an industrial scale particularly advantageously under economic and ecological aspects.

This object is achieved by a process with the characterizing features of claim 1 of the present invention. Claims 2 to 7 relate to preferred embodiments. Claims 8 and 9 protect a reaction system according to the invention and a correspondingly operating whole cell catalyst. Claim 10 protects preferred plasmids.

In a process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, as a result of not employing the pyruvate formed from the second enzymatic transformation as the substrate in the first enzymatic transformation, the object described is achieved in an entirely surprising but in return no less advantageous manner. It can be regarded entirely as surprising that the simultaneous use e.g. of an amino acid dehydrogenase with malate dehydrogenase, which has a decarboxylating action, is possible without problems without the occurrence of cross-reactions. In addition, no inhibiting influences of substrates and products of the primary reaction on the malate dehydrogenase and vice versa can be detected. In particular, it is a positive feature that the pyruvate formed as the by-product does not have an inhibiting action on the malate dehydrogenase itself or on the alcohol or amino acid dehydrogenases employed in parallel.

Enantiomerically enriched alcohols or amino acids are advantageously prepared with the process according to the invention. In this case, inexpensive alcohol dehydrogenase or amino acid dehydrogenase which are known universally are possible as enzymes for the first enzymatic transformation. The expert is in principle free in the choice thereof, which is made according to the nature of the substrate spectrum, stability and rate of conversion of the enzyme in question. Known enzymes of this origin are described in K. Drauz, H. Waldmann (eds.), Enzyme Catalysis in Organic Synthesis, volume III, Wiley-VCH, Weinheim, **2002**, chapter 15.

The use of the alcohol dehydrogenase from the organisms *Rhodococcus erythropolis* (S-ADH) or *Lactobacillus kefir* (R-

ADH) (ADH from *R. erythropolis*: J. Peters, T. Zelinski, M.-R. Kula, Purification and characterization of a novel carbonyl reductase silated from *Rhodococcus erythropolis*, J. Biotechnol. 1994, 33, 283-292)(ADH from *Lactabacillus kefir*: C. W. Bradshaw, W. Hummel, C.-H. Wong, *Lactobacillus kefir* Alcohol Dehydrogenase: A Useful Catalyst for Synthesis, J. Org. Chem. 1992, 57, 1532-1536.) is particularly advantageous. In respect of preferred amino acid dehydrogenases, the expert is referred to, for example, leucine dehydrogenases or phenylalanine dehydrogenases (A. Bommarius in: Enzyme Catalysis in Organic Synthesis (eds.: K. Drauz, H. Waldmann), volume III, Wiley-VCH, Weinheim, **2002**, chapter 15.3).

Malate dehydrogenases are also familiar to the expert (lit. see above or the dissertation by S. Naamnieh, University of Düsseldorf, in preparation). Here also, the expert will choose the dehydrogenase which can be employed most efficiently for his purpose. In principle, those malate dehydrogenases which regenerate the NAD(P)H in an extent such that no bottleneck arises for the course of the reaction of the other enzyme employed.
The known malate dehydrogenase from *E. coli* K12 is preferred in this connection. Gene isolation and cloning are described in S. Naamnieh's dissertation, University of Düsseldorf, in preparation p. 70 et seq.

In principle, the process according to the invention can be carried out in purely aqueous solution. However, it is also possible to add any desired parts of a water-soluble organic solvent to the aqueous solution in order e.g. to optimize the reaction in respect of poorly water-soluble substrates. Possible such solvents are, in particular, ethylene glycol, DME or glycerol.
However, multi-phase, in particular two-phase systems comprising an aqueous phase can furthermore also serve as the solvent mixture for the process according to the

invention. The use of certain solvents which are not water-soluble has already proved itself here (DE10233107). The statements made there in this respect also apply here accordingly.

The expert in principle has a free choice of the temperature present during the reaction. He preferably directs himself towards obtaining a highest possible yield of product in the highest possible purity in the shortest possible time. Furthermore, the enzymes employed should be sufficiently stable under the temperatures employed and the reaction should proceed with the highest possible enantioselectivity. In respect of the use of enzymes from thermophilic organisms, it is entirely possible for temperatures of 100°C to represent the upper limit of the temperature range in the reaction. -15°C is certainly appropriate as the lower limit in aqueous systems. A temperature interval between 10 and 60, particularly preferably between 20 and 40°C is advantageously to be established.

The pH during the reaction is determined by the expert from the enzyme stabilities and conversion rates and is adjusted accordingly for the process according to the invention. For the malate dehydrogenase from *E. coli* it has been found that the optimum pH is > 10. In general, the range preferred for enzymes from pH 5 to 11 is chosen. A pH range from 5.5 to 10.0, in particular 6.0 to 9.0, can preferably be present.

The invention also provides a coupled enzymatic reaction system for the preparation of enantiomerically enriched organic compounds, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, wherein the pyruvate formed from the second enzymatic transformation is

not employed as the substrate in the first enzymatic transformation. The same advantages and preferred embodiments as have already been mentioned in respect of the process according to the invention apply in principle to this reaction system.

The reaction system is advantageously employed, for example, in a stirred tank, a cascade of stirred tanks or in membrane reactors, which can be operated both in batch operation and continuously.

In the context of the invention, membrane reactor is understood as meaning any reaction vessel in which the catalyst is enclosed in a reactor, while low molecular weight substances are fed to the reactor or can leave it. The membrane here can be integrated directly into the reaction space or incorporated outside in a separate filtration module, in which the reaction solution flows continuously or intermittently through the filtration module and the retained product is recycled into the reactor. Suitable embodiments are described, inter alia, in WO98/22415 and in Wandrey et al. in Yearbook 1998, Verfahrenstechnik und Chemieingenieurwesen, VDI p. 151 et seq.; Wandrey et al. in Applied Homogeneous Catalysis with Organometallic Compounds, vol. 2, VCH 1996, p. 832 et seq.; Kragl et al., Angew. Chem. 1996, 6, 684 et seq.

The continuous procedure which is possible in this apparatus, in addition to the batch and semi-continuous procedure, can be carried out here as desired in the cross-flow filtration mode (fig. 3) or as dead-end filtration (fig. 2). Both process variants are described in principle in the prior art (Engineering Processes for Bioseparations, ed.: L.R. Weatherley, Heinemann, 1994, 135-165; Wandrey et al., Tetrahedron Asymmetry 1999, 10, 923-928).

The present invention also provides whole cell catalysts comprising a cloned gene for a first enzyme for transformation of an organic substrate and a cloned gene for a malate dehydrogenase, these being capable of

preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, NAD(P)H being consumed, and of allowing the regeneration of the NAD(P)H to take place in a second enzymatic transformation by malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, wherein the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

The whole cell catalyst according to the invention preferably has an enzyme (polypeptide) with amino acid or alcohol dehydrogenase activity and one with malate dehydrogenase activity, originating in particular from the organisms mentioned above.

Microorganisms which can be used are in principle all the organisms possible to the expert for this purpose, such as e.g. yeasts, such as *Hansenula polymorpha*, *Pichia sp.* and *Saccharomyces cerevisiae*, prokaryotes, such as *E. coli* and *Bacillus subtilis* or eukaryotes, such as mammalian cells and insect cells. *E. coli* strains are preferably to be used for this purpose. The following are very particularly preferred: *E. coli* XL1 Blue, NM 522, JM101, JM109, JM105, RR1, DH5α, TOP 10- or HB101.

An organism as mentioned in DE10155928 is preferably employed as the host organism.

The advantage of such an organism is the simultaneous expression of both polypeptide systems, which means that only one rec-organism has to be cultured for the reaction according to the invention.

To coordinate the expression of the polypeptides in respect of their rates of conversion, the corresponding coding nucleic acid sequences can be accommodated on different plasmids with different numbers of copies and/or promoters of different potency can be used for an expression of the nucleic acid sequences of different intensity. In such coordinated enzyme systems, advantageously no accumulation of an intermediate compound occurs, and the reaction in question can proceed at an optimum overall rate. However,

this is adequately known to the expert (Gellissen, G.; Piontek, M.; Dahlems, U.; Jenzelewski, V.; Gavagan, J. W.; DiCosimo, R.; Anton, D. L.; Janowicz, Z. A. (1996), Recombinant Hansenula polymorpha as a biocatalyst. Coexpression of the spinach glycollate oxidase (GO) and the S. cerevisiae catalase T (CTT1) gene, Appl. Microbiol. Biotechnol. 46, 46-54; Farwick, M.; London, M.; Dohmen, J.; Dahlems, U.; Gellissen, G.; Strasser, A. W.; DE19920712).

It is very particularly advantageous that the whole cell catalyst according to the invention optionally further metabolizes the pyruvate formed in the reaction according to the invention, as it may uses it as a nutrient source. Whole cell catalysts designed in this manner mean that the pyruvate is not obtained as a by-product of the reaction and therefore also does not have to be separated off from the chiral product actually desired in further process steps.

The preparation of the whole cell catalyst can in principle be carried out by measures known to the expert (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York; Balbas, P. and Bolivar, F. (1990), Design and construction of expression plasmid vectors in E. coli, Methods Enzymol. 185, 14-37; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 205-225, Butterworth, Stoneham). In respect of the general procedure (PCR, cloning, expression etc.) reference may also be made to the following literature and that cited there: Universal GenomeWalker™ Kit User Manual, Clontech, 3/2000 and literature cited there; Triglia T.; Peterson, M. G. and Kemp, D.J. (1988), A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences, Nucleic Acids Res. 16, 8186; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York; Rodriguez, R.L. and Denhardt, D. T (eds)

(1988), Vectors: a survey of molecular cloning vectors and their uses, Butterworth, Stoneham.

The invention also provides plasmids containing gene constructs in which the gene for a malate dehydrogenase and a gene for an enzyme for transformation of an organic substrate with consumption of NAD(P)H are present. Possible plasmids or vectors of origin are in principle all the embodiments available to the expert for this purpose. Such plasmids and vectors can be found e.g. in Studier and colleagues (Studier, W. F.; Rosenberg A. H.; Dunn J. J.; Dubendroff J. W.; (1990), Use of the T7 RNA polymerase to direct expression of cloned genes, Methods Enzymol. 185, 61-89) or the brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D. M. (1985), DNA cloning: A Practical Approach, vol. I-III, IRL Press Ltd., Oxford; Rodriguez, R.L. and Denhardt, D. T (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, Butterworth, Stoneham; Goeddel, D. V. (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7; Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989), Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York.

Plasmids with which the gene construct containing the nucleic acids according to the invention can be cloned into the host organism in a very preferred manner are: pUC18 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia Biotech), pKK-233-3 (Stratagene) or pET (Novagen). The plasmid pkk/phe/mali (fig. 5) is advantageous in this connection.

The preparation of this plasmid and of a corresponding rec-microorganism is described in the dissertation by S. Naamnieh, University of Düsseldorf, in preparation p. 70 et seq.

For the use, the polypeptides in question of the process according to the invention can be used in the free form as homogeneously purified compounds or as an enzyme prepared by a recombinant method. These polypeptides can furthermore also be employed as a constituent of an intact guest organism or in combination with the broken-down cell mass of the host organism, which has been purified to any desired extent.

The use of the enzymes in immobilized form is also possible (Sharma B. P.; Bailey L. F. and Messing R. A. (1982), Immobilisierte Biomaterialiern - Techniken und Anwendungen, Angew. Chem. 94, 836-852). The immobilization is advantageously carried out by lyophilization (Paradkar, V. M.; Dordick, J. S. (1994), Aqueous-Like Activity of α-Chymotrypsin Dissolved in Nearly Anhydrous Organic Solvents, J. Am. Chem. Soc. 116, 5009-5010; Mori, T.; Okahata, Y. (1997), A variety of lipi-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents, Tetrahedron Lett. 38, 1971-1974; Otamiri, M.; Adlercreutz, P.; Matthiasson, B. (1992), Complex formation between chymotrypsin and ethyl cellulose as a means to solubilize the enzyme in active form in toluene, Biocatalysis 6, 291-305). Lyophilization in the presence of surface-active substances, such as Aerosol OT or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Kamiya, N.; Okazaki, S.-Y.; Goto, M. (1997), Surfactant-horseradish peroxidase complex catalytically active in anhydrous benzene, Biotechnol. Tech. 11, 375-378), is very particularly preferred.

Immobilization on Eupergit®, in particular Eupergit C® and Eupergit 250L® (Röhm) (Eupergit.RTM. C, a carrier for immobilization of enzymes of industrial potential. Katchalski-Katzir, E.; Kraemer, D. M. Journal of Molecular Catalysis B: Enzymatic (2000), 10(1-3), 157-176), is extremely preferred.

Immobilization on Ni-NTA in combination with the

polypeptide supplemented with the His tag (hexa-histidine) is likewise preferred (Purification of proteins using polyhistidine affinity tags. Bornhorst, Joshua A.; Falke, Joseph J. Methods in Enzymology (2000), 326, 245-254). The use as CLECs is also conceivable (St. Clair, N.; Wang, Y.-F.; Margolin, A. L. (2000), Cofactor-bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase, Angew. Chem. Int. Ed. 39, 380-383).

By these measures it can be possible to generate from polypeptides which become unstable due to organic solvents those which can operate in mixtures of aqueous and organic solvents or entirely in organic media.

The process according to the invention can be carried out such that the MDH from *E. coli* is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH (equation (1)).

$$\text{2-keto-isocaproate} + NADH + NH_4^+ \longrightarrow \text{L-leucine} + NAD^+ \qquad (1a)$$

$$NAD^+ + \text{L-malate} \longrightarrow \text{pyruvate} + CO_2 + NADH \qquad (1b)$$

The course of the reaction was monitored by HPLC. The result can be seen from the following table 1 in comparison with an equivalent conversion with FDH instead of MDH.

Table 1: Comparison of the formation of L-leucine (HPLC) in a coupled batch with coenzyme regeneration by MDH and FDH. In each case 10 mM ketoisocaproate and for the regeneration by malate dehydrogenase (MDH) 100 mM L-malate or by formate dehydrogenase (FDH) 100 mM formate were employed.

Time [min]	L-Leucine with MDH [mM]	L-Leucine with FDH [mM]
0	0.7	0.1
10	8.8	9.9
30	9.6	11.1
60	10.8	9.8
120	9.9	9.9

The use of alcohol dehydrogenases in combination with malate dehydrogenases was also investigated. The MDH from *E. coli* is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH; DE10218689). The usability of the MDH is tested here via the reduction of a ketone (p-Cl-acetophenone = pCAp) in accordance with equation (4).

$$\text{pCAp} + \text{NADH} + H^+ \longrightarrow \text{p-Cl-phenylethanol} + \text{NAD}^+ \quad (4a)$$

$$\text{NAD}^+ + \text{L-malate} \longrightarrow \text{pyruvate} + CO_2 + \text{NADH} \quad (4b)$$

Table 2: Decrease in the ketone p-Cl-acetophenone (employed 10 mM) and increase in the enzymatically formed alcohol p-Cl-phenylethanol (100%, corresp. to 10 mM) as a function of time.

Time [min]	Ketone [%]	Alcohol [%]
0	100	0
10	28	72
20	12	88
30	4	96
60	0	100

Enantiomerically enriched or enantiomer-enriched describes the fact that one optical antipode is present in a mixture with its other to >50%.

If one stereo-centre is present the structures shown relate to the two possible enantiomers, and if more than one stereo-centre is present in the molecule they relate to all the possible diastereomers and, in respect of a diastereomer, to the two possible enantiomers of the compound in question which fall under this.

The organism *Candida boidinii* is deposited under number ATCC 32195 at the American Type Culture Collection and is accessible to the public.

The documents of the prior art mentioned in this specification are regarded as also included in the disclosure.

Descriptions of the drawings:

Fig. 2 shows a membrane reactor with dead-end filtration. The substrate 1 is transferred via a pump 2 into the reactor space 3, which contains a membrane 5. In the reactor space, which is operated with a stirrer, are, in addition to the solvent, the catalyst 4, the product 6 and unreacted substrate 1. Low molecular weight 6 is chiefly filtered off via the membrane 5.

Fig. 3 shows a membrane reactor with cross-flow filtration. The substrate 7 is transferred here via the pump 8 into the stirred reactor space, in which is also solvent, catalyst 9 and product 14. A solvent flow which leads via a heat exchanger 12, which may be present, into the cross-flow filtration cell 15 is established via the pump 16. The low molecular weight product 14 is separated off here via the membrane 13. High molecular weight catalyst 9 is then passed back with the solvent flow, if appropriate via a heat exchanger 12 again, if appropriate via the valve 11, into the reactor 10.

Example 1:

For production of the MDH from *E. coli* K12 used here, see the dissertation by S. Naamnieh, University of Düsseldorf, in preparation, p. 70 et seq.

Purification and biochemical properties of the MDH from E. coli:

a) Purification

The purification of the recMDH from *E. coli* crude extracts (expression strain: *E. coli* derivative JM105) was carried out in accordance with purification protocol (Stols L., and Donnelly M. I. (1997). Production of succinic acid through overexpression of NAD(+)-dependent malic enzyme in an Escherichia coli mutant. *Appl Environ Microbiol* **63**: 2695-701.). The rec-bacteria cells were first broken down by disintegration with glass beads (breakdown buffer Tris/HCl 100 mM pH 7.5). Thereafter, a purification step by a Q-Sepharose was carried out. After the purification by means of Q-Sepharose, it was possible to determine a specific activity of the MDH of about 7.3 U/mg. By purification of this enzyme by means of further chromatography steps, hydroxyapatite and Phenylsepharose, it was possible to purify the MDH to homogeneity with a specific activity of 133 U/mg.

Tab. 3: Summary of the purification of the rec-MDH from *E. coli*.

Purification step	Activity (U)	Protein (mg)	Spec. act. (U/mg)	Enrichment (-fold)	Yield (%)
Crude extract	210	202	1.03		100
Q-Sepharose	184	25.2	7.3	7.1	88
Hydroxy-apatite	68	1.6	42.5	41	32
Phenyl-sepharose	24	0.18	133	129	11

b) Biochemical characterization

- Km values

For L-malate a Km value of 0.29 mM was measured, and for the coenzyme NAD^+ a Km value of 0.14 mM.
Both Km values lie in a low range of < 1 mM, and they show that the two substrates are recognized by the enzyme with a good affinity. The two values suggest that the MDH can be used for the regeneration of NADH.

- Optimum pH (fig. 1)

MDH shows a maximum activity at relatively high pH values of 11 and higher. Nevertheless, the drop in activity at lower pH values is relatively small, thus 72% activity is still present at pH 8.0, and 67% activity still at pH 7.0.

- Optimum temperature

The optimum temperature of the MDH is approx. 55°C (fig. 4).

Example 2:

a) Coupling of leucine dehydrogenase with malate dehydrogenase

The MDH from E. coli is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus;* Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH.

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses):

526 µl Hepes buffer (200 mM Hepes, pH 8.5 with 10 mM $MgCl_2$); 143 µl ammonium sulfate solution (500 mM in the test); 100 µl ketoisocaproate (100 mM); 20 µl NAD^+ (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 1 µl LeuDH (0.5 U in the test); 10 µl MDH (1.5 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 30, 60 and 120 min samples are taken (50 µl; Eppendorf reaction vessels) and heated for 3 min at 95°C to stop the reaction. Denatured protein is separated off by centrifugation for 10 min at 13,000 rpm (Eppendorf bench centrifuge) and the supernatant is analysed by means of HPLC, after derivatization with ortho-phthalaldehyde (OPA).

Derivatization with OPA (= ortho-phthaldialdehyde):

140 µl Na borate buffer (100 mM; pH 10.4); 40 µl sample or standard; 20 µl OPA/IBLC reagent (= ortho-phthaldialdehyde

/ N-isobutyryl-L-cysteine). 20 µl of this reaction solution are injected for the HPLC analysis.

HPLC analysis:

For the results see table 1:

b) Comparison experiment: Coupling of leucine dehydrogenase with formate dehydrogenase

In a comparison batch carried out in parallel, the same components as described above were used, but instead of the malate dehydrogenase 0.5 U formate dehydrogenase (FDH from *Candida boidinii;* Sigma) was employed and instead of 100 mM L-malate 100 mM formate was used as the regeneration substrate.

For the results see table 1.

Example 3: Coupling of the MDH with alcohol dehydrogenase:

The MDH from *E. coli* (expression strain: *E. coli* derivative JM 105) is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH). The usability of the MDH is tested here via the reduction of a ketone (p-Cl-acetophenone).

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses):

678.7 µl Hepes buffer (100 mM Hepes, pH 8.5 with 10 mM $MgCl_2$); 1.3 µl p-Cl-acetophenone (10 mM in the test); 20 µl NAD^+ (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 15 µl RE-ADH (1 U in the test); 85 µl MDH (1 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 20, 30 and 60 min samples (50 µl) are taken, 100 µl ethyl acetate are added and the upper phase is analysed by means of gas chromatography for the formation of the alcohol p-Cl-phenylethanol.

For the results see table 2.

Example 4: Construction of an expression vector with heterologous expression

From the sequence of the amplified fragment, primers with integrated restriction cleavage sites and a codon for the ribosomal binding site were constructed. After amplification of the malate dehydrogenase from the recombinant pUC18, the PCR fragment was cloned into the recombinant recPhe-pKK-223-3 expression vector after the PheDH sequence at the PstI and HindIII restriction cleavage sites (fig. 8 - Construction of the plasmid for a heterologous expression for L-Phe synthesis by means of whole cell conversion).

PCR:

5' forward: N'-malic-pst
5' CTGCAGAGCCCAGGGATGGATATTCAAAAA 3'
concentration 100 pmol/µl

5' reverse: C'-malic-Hin
5' AAGCTTTTAGATGGAGGTACGGCGGTAGTC 3'
concentration 100 pmol/µl

Table 4: PCR protocol for amplification of the malate dehydrogenase from the recombinant pUC18 plasmid. The concentrations of the template DNA were varied.

Template DANN recpUC18	N' -malic-pst prim 1	C' -malic-Hin prim 2	dNTP	Buffer	Taq polymerase	H_2O
50 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl
25 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl
10 ng/µl	1 µl	1 µl	2 µl	10 µl	1 µl	83 µl

One cycle consists of:

Denaturing step : 94°C
Annealing step: 59°C
Amplification step: 72°C

Cloning:

The new construct of the recombinant plasmid (fig. 5) was transformed into competent *E. coli* cells JM 105 or HB 101.

The standard transformation was carried out in accordance with the protocol of Hanahan (Hanahan D. (1983). Studies on transformation of Escherichia coli with plasmids. *J Mol Biol* **166:** 557-80.). For this, 100-200 µl of competent *E. coli* cells were thawed on ice and 40 ng DNA from the ligation batch were added. The plasmid cell suspension was cooled for 30 min on ice and then heated at 42ºC for 90 sec and immediately cooled again on ice. After addition of 300 µl LB medium the cells were incubated for about 45 min at 37ºC for regeneration. 200 µl of this culture were then

plated out on to an LB plate containing antibiotic and incubated overnight at 37ºC.

With the cloning of the malate dehydrogenase 3' to the PheDH on the PstI and HindIII cleavage site with its own ribosomal binding site, expression of the two enzymes could take place simultaneously.
Note: Further detailed information on the experimental preparation of these cells will be described in: S. Naamnieh, *Dissertation*, University of Düsseldorf, in preparation.

Example 5: Coexpression of PheDH and malate dehydrogenase

Of the positive clones, some were selected for expression An individual colony of the particular clones was transinoculated into 5 ml LB_{amp} medium, and after OD_{580} = 0.6 was reached was induced with 1 mM IPTG. The induction was carried out overnight and the harvested cells were broken down with ultrasound.

The recombinant strain HB101 shows a malate dehydrogenase activity of 100 U/ml and likewise a PheDH activity of 130 U/ml. The activity of the two enzymes in the recombinant strain JM 105 is clearly higher and is at ~ 600 U/ml for malate dehydrogenase and ~ 1,200 U/ml for the PheDH.

The two recombinant strains were cultured in a 10 l fermenter and the activity of the two enzymes was determined.

Table 5: Determination of the activity of the expressed enzymes in a 10 l fermenter with LB medium as batch fermentation.

	Activity of PheDH		Activity of malate dehydrogenase	
	(U/ml)	(U/mg)	(U/ml)	(U/mg)
E. coli HB101	400	33	220	22
E. coli JM105	1300	118	640	71

It can be seen from the expression data that the *E. coli* strain JM 105 shows a significantly better activity for both enzymes, and all further experiments were therefore carried out with this strain.

Example 6: Optimization of the activity

To be able to utilize the maximum activity of the crude extracts obtained, several parameters were investigated and varied.

The two heterologously expressed enzymes show the best stability properties under different conditions. It was of interest to determine the optimum properties for both enzymes in the same system. The following experiments were carried out with a view to this fact.

a) Optimization of the breakdown buffer

1 g of cells JM_{105} was broken down in 0.1 M Tris or 0.1 M Kpi buffer with/without BSA (1.5 g/l) 30%. The breakdown was carried out with ultrasound over 70 cont. cycles. In

addition to the buffers, 1.5 g/l BSA were added for stabilization of the enzymes.

Table 6: Comparison of the activity as a function of the breakdown buffer

	0.1 M Tris		0.1 M Kpi	
	- BSA	+ BSA	- BSA	+ BSA
PheDH	520 U/ml	610 U/ml	730 U/ml	1100 U/ml
Malate dehydrogenase	430 U/ml	720 U/ml	320 U/ml	610 U/ml

The addition of BSA led to an increase in the activity in both cases. The breakdown buffer also influenced the activities. It was to be seen here that the suitable breakdown buffer was different for the individual enzymes. The Kpi buffer was more suitable for the PheDH than for the malate dehydrogenase, but since the decrease in activity of the malate dehydrogenase in the Kpi buffer was relatively low, the recombinant cells continued to be broken down in this buffer after the heterologous expression.

b) Duration of breakdown to investigate the stability

The duration of the breakdown was also investigated and the critical point for stability of the enzymes during this operation was determined. The optimum duration of breakdown can be obtained from these data (fig. 9 - Stability determination of the PheDH and the malate dehydrogenase after various breakdown times by means of ultrasound. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

The following suspension was used for this experiment:

1 g recombinant cells (JM105)
3 ml Kpi buffer 0.1 M

During longer treatment of the cells with ultrasound the activity of the PheDH decreases drastically, whereas the activity of the malate dehydrogenase is retained. The ideal breakdown conditions for a 25% breakdown of 1 g of recombinant *E. coli* JM105 are therefore 4 x 30 s ultrasound treatment with 3 x 30 s intermediate cooling in an ice-bath.

During longer treatments with ultrasound the sample is heated, which can lead to denaturing of the enzymes. The amount of protein was determined in this experiment and can be seen from the following.

Table 7: Protein determination of the two enzymes expressed, PheDH and malate dehydrogenase, after variation of the breakdown time. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

	60 sec x 2 spec. activity: U/mg	30 sec x 4 spec. activity: U/mg	30 sec x 8 spec. activity: U/mg
PheDH	30	105	16
Malate dehydrogenase	20	90	110

With a purification of the malate dehydrogenase to homogeneity, it was possible to achieve a specific activity of 466 U/mg. The purification steps are summarized in table 8.

Table 8: Purification of the recombinant malate dehydrogenase

	Volume (ml)	Activity (U)	Protein (mg)	Spec. activity (U/mg)	Yield (%)	Factor
Ultracen-trifugation	0.6	820	13	63	100	1
Hydroxy-apatite	9	470	5	94	57	1.49
Q-Sepharose	7	496	2.1	236	60	3.74
Phenyl-sepharose	1.9	280	0.6	466	34	7.39

c) Km value determination

The K_M values were determined for the substrate and the coenzyme of the malate dehydrogenase. The K_M values were determined on homogeneous or partly purified rec-malate dehydrogenase samples.

L-Malate:	0.29 mM
NAD^+	0.14 mM

Example 7: Coupled L-phenylalanine synthesis with regeneration of the coenzyme NADH

Important factors for a coupled reaction are the optimum pH and the heat stability of the two enzymes. In addition to the stability of the enzymes, further factors play a role, such as e.g. the influence of the various substrates on the enzymes.

In respect of the optimum pH, a kinetics study was conducted and the pH-dependency of the synthesis was determined. The increase in the activity at an increasing pH can be seen from fig. 10, a pH of 8.0, in which although the two enzymes do not show the highest activity, the coenzyme remains stable, being chosen for the synthesis for coenzyme stability reasons (fig. 10 - Optimum pH of malate dehydrogenase and PheDH. The activity of the two enzymes increases as the pH increases. The measurements were carried out with partly purified enzyme. For the phenylalanine dehydrogenase, the reductive amination was measured).

A second factor for the coupled enzyme reaction is the suitable temperature at which the two enzymes remain stable for a relatively long period of time. A further experiment was therefore carried out to determine the optimum temperature (fig. 11 - Optimum temperature. The measurements were carried out at pH 8.5 and in 0.1 M HEPES buffer).

As can be seen from fig. 11, the optimum temperature of both enzymes is 50°C. At 30°C the activity measured is only 60%.

The malate dehydrogenase is stable at 45°C for a relatively long period of time. However, since the PheDH becomes unstable at this temperature value, the syntheses were carried out at 30°C, so that it was possible to ensure the stability of both enzymes and of the coenzyme over a relatively long period of time.

Enzymes achieve their optimum activity in the particular suitable buffer. The two enzymes were tested with two different buffers in each case in a reaction batch (table 9).

Table 9: Comparison of the PheDH and malate dehydrogenase activity in various reaction buffers. The activities are to be seen in per cent of the optimum.

	0.1 M HEPES buffer (pH 8.0)	0.1 M Tris buffer (pH 8.0)
PheDH	84 %	100 %
MDH	100 %	42 %

Since the activity of the PheDH in HEPES buffer does not decrease considerably, the coupled enzyme reaction was carried out in this buffer.

With the determination of the buffer, pH and temperature values, it was possible to select suitable conditions and media for the synthesis of phenylalanine by a coupled enzyme reaction with regeneration of the cofactor (NADH).

30 mM phenyl pyruvate, 100 mM ammonium sulfate, 100 mM HEPES buffer, 70 mM L-malate, 2 mM NAD^+, 2 mM Mg^{2+}, 25 U PheDH (partly purified) and 30 U malate dehydrogenase were employed. The samples are analysed by means of HPLC. The synthesis was monitored for several hours. After 4 h approx. 50% of the substrate employed, phenyl pyruvate, was converted into L-phenylalanine (fig. 12 - Formation kinetics for L-Phe. The formation of L-Phe was carried out *in situ*).

Example 8: Whole cell conversion

The following medium was used as the standard batch for the whole cell conversion:

0.1 M	HEPES buffer (pH 8.0)
40 mM	phenyl pyruvate
0.1 M	L-malate
0.1 M	ammonium sulfate
2 mM	$MgCl_2$

The conversion was carried out at 30°C and with 1 g of recombinant *E. coli* cells. The phenylalanine formed was detected by means of HPLC (fig. 6).

The formation of L-Phe by recombinant *E. coli* cells was monitored for 20 h and the yield was determined (fig. 7).

No metabolization of the product formed, L-Phe, was to be detected after incubation for 20 h.

Patent claims:

1. Process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

2. Process according to claim 1, characterized in that the first enzymatic transformation proceeds using an alcohol dehydrogenase or amino acid dehydrogenase.

3. Process according to claim 2, characterized in that an ADH from *Lactobacillus kefir* or *Rhodococcus erythropolis* is used as the alcohol dehydrogenase and a leucine dehydrogenase or phenylalanine dehydrogenase is used as the amino acid dehydrogenase.

4. Process according to one or more of the preceding claims characterized in that a malate dehydrogenase from *E. coli*, in particular *E. coli* K12, is used.

5. Process according to one or more of the preceding claims characterized in that the reaction is carried out in an aqueous single- or multi-phase solvent mixture.

6. Process according to one or more of the preceding claims, characterized in that the temperature during the reaction is between 20 and 40°C.

7. Process according to one or more of the preceding claims, characterized in that the pH during the reaction is between 6 and 9.

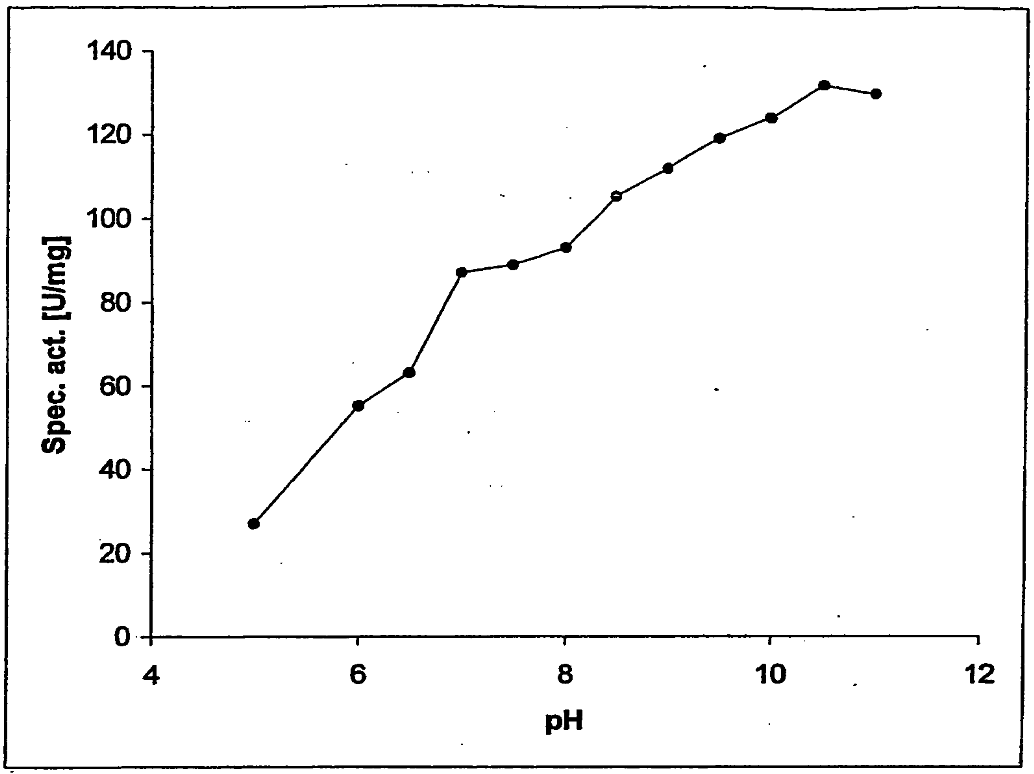
8. Coupled enzymatic reaction system for the preparation of enantiomerically enriched organic compounds, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

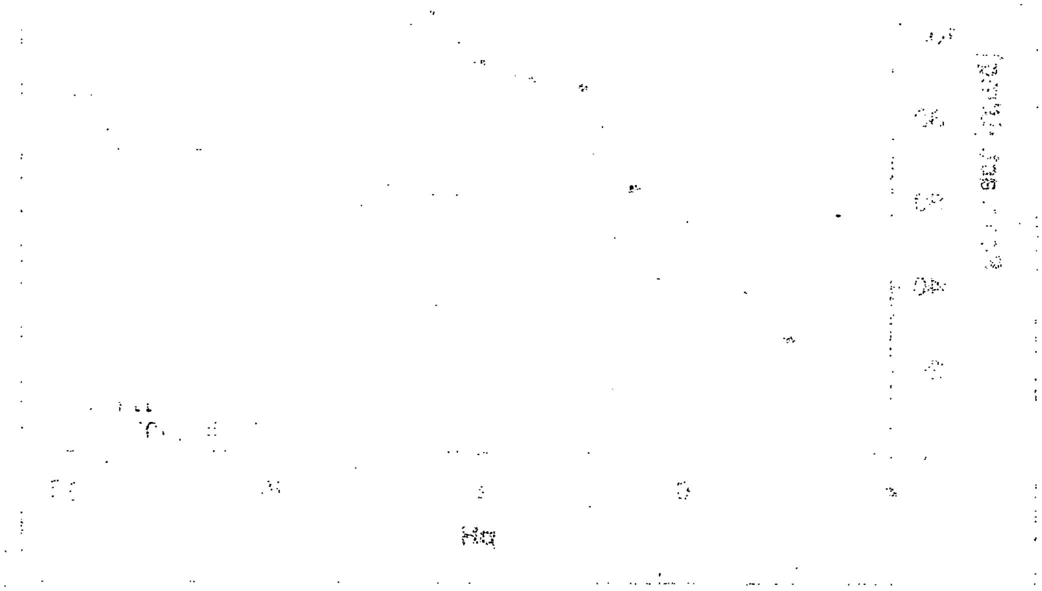
9. Whole cell catalyst comprising a cloned gene for a first enzyme for transformation of an organic substrate and a cloned gene for a malate dehydrogenase, this being capable of preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, NAD(P)H being consumed, and of allowing the regeneration of the NAD(P)H to take place in a second enzymatic transformation by malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO_2, wherein the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

10. Plasmids containing gene constructs in which the gene for a malate dehydrogenase and a gene for an enzyme

for transformation of an organic substrate with consumption of NAD(P)H are present.

Fig. 1:





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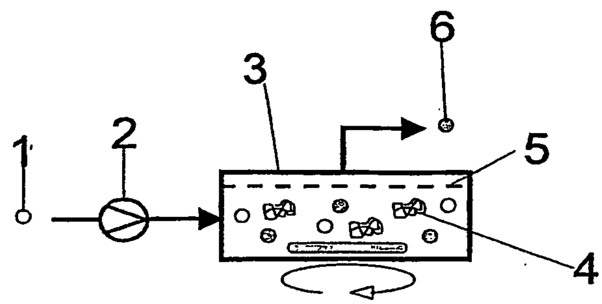
6
3
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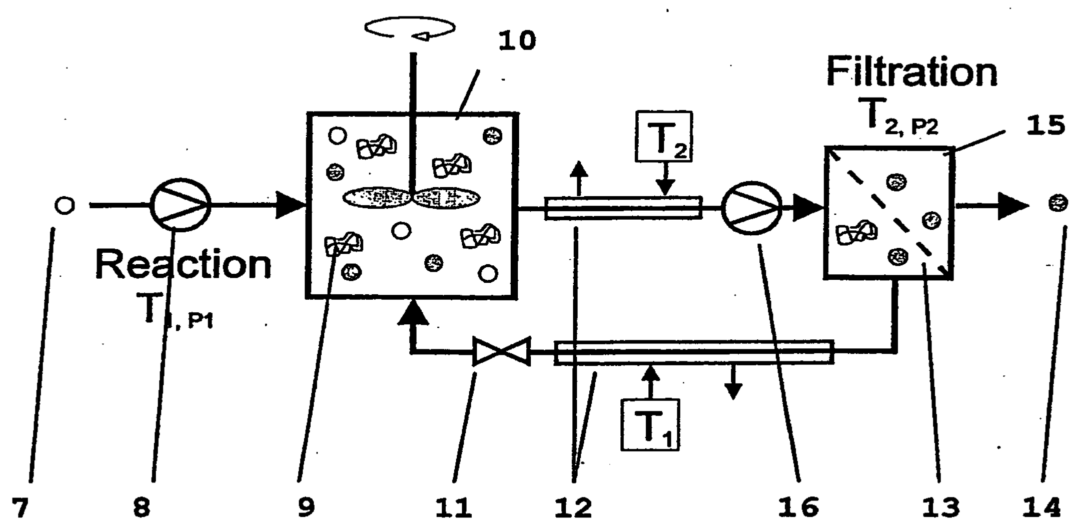
1
5
4

Fig. 3:


10
Filtration
$T_{2,\ P2}$
15
T_2
Reaction
$T_{1,\ P1}$
T_1
7
8
9
11
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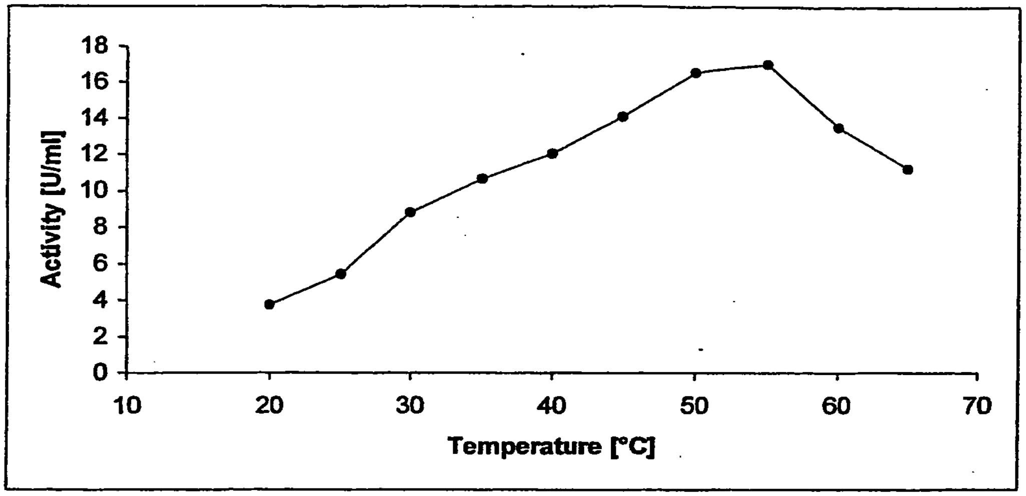
16
13
14

Fig. 4:


Activity [U/ml]
18
16
14
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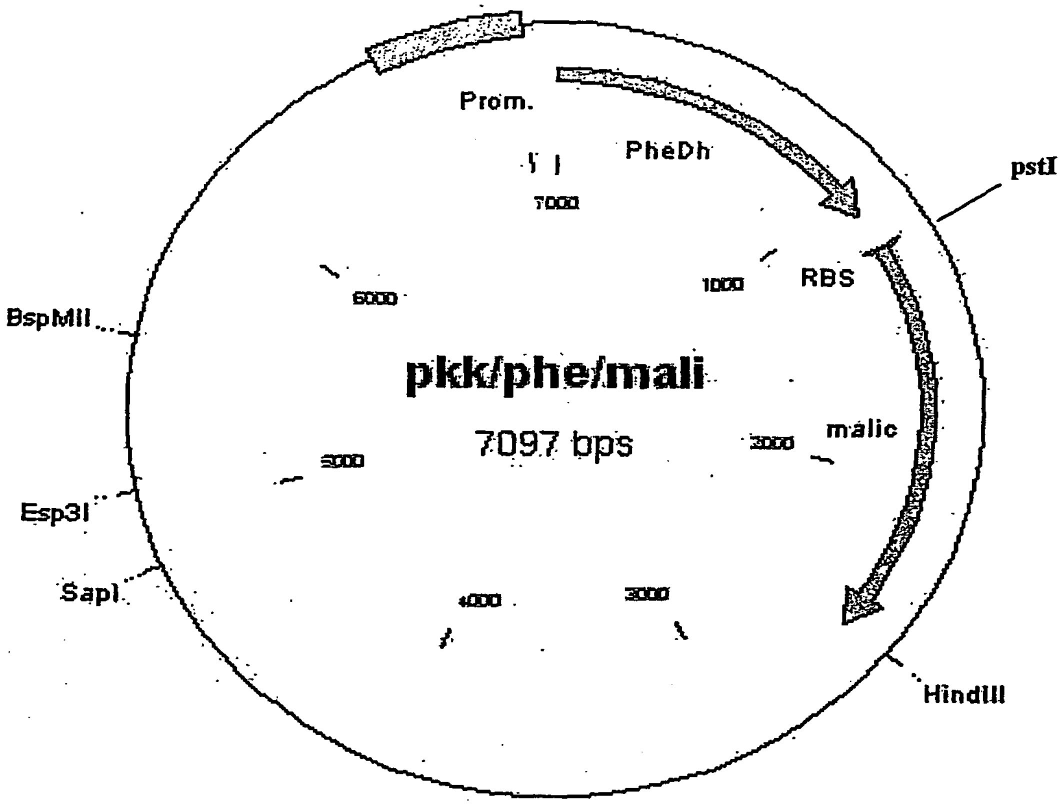
10
8
6
4
2
0
10
20
30
40
50
60
70
Temperature [°C]

Fig. 5: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PheDh
pstI
7000
1000
RBS
6000
BspMII
pkk/phe/mali
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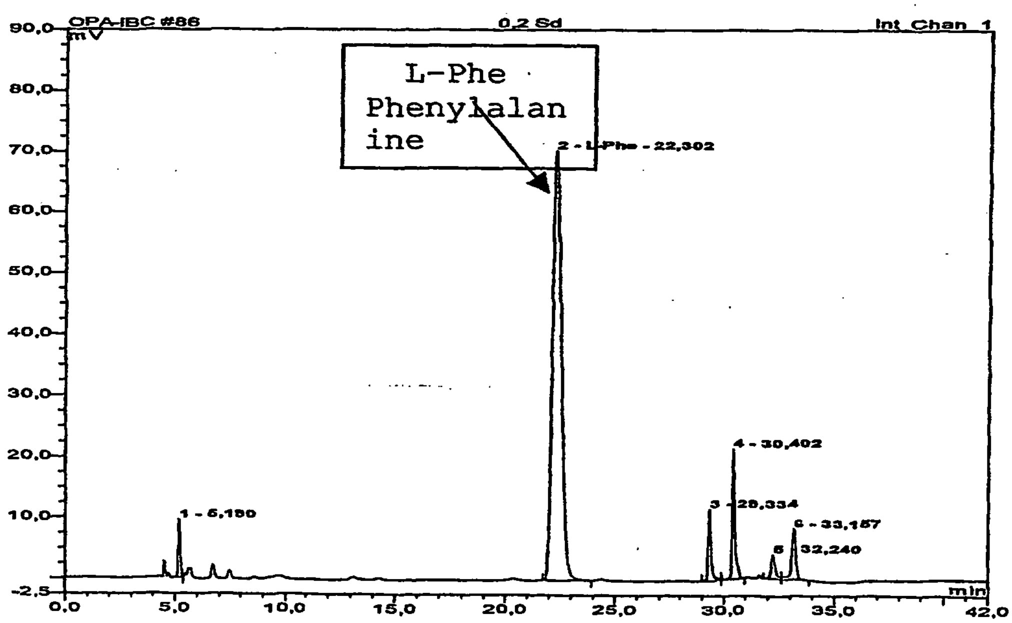
malic
5000
Esp3I
SapI
4000
3000
HindIII

Fig. 6:


OPA-IBC #86
0,2 Sd
Int_Chan_1
mV
L-Phe
Phenylalanine
2 - L-Phe - 22,302
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1 - 5,150
5 32,240
6 - 33,157
min

Fig. 7:

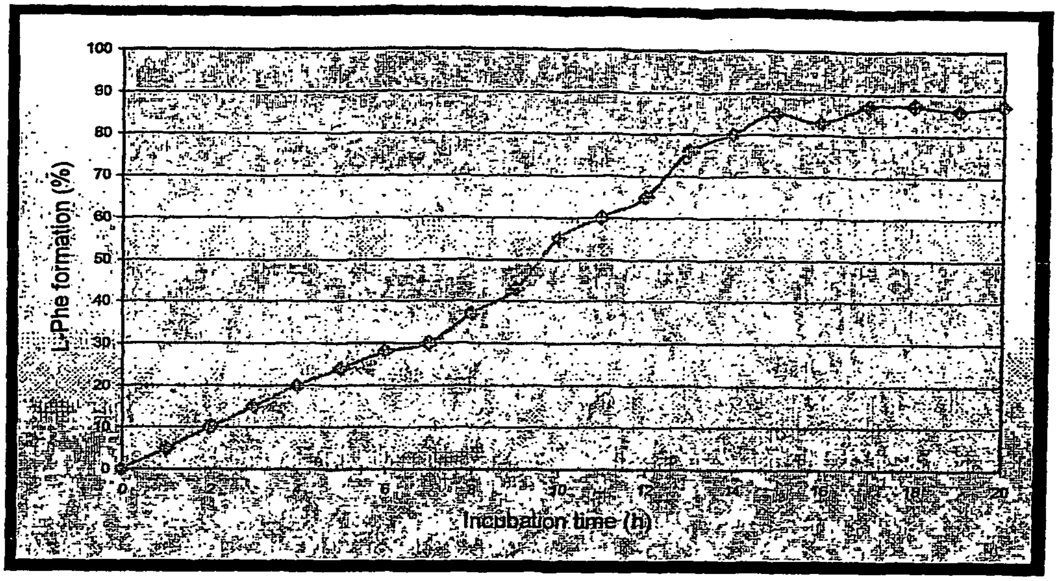
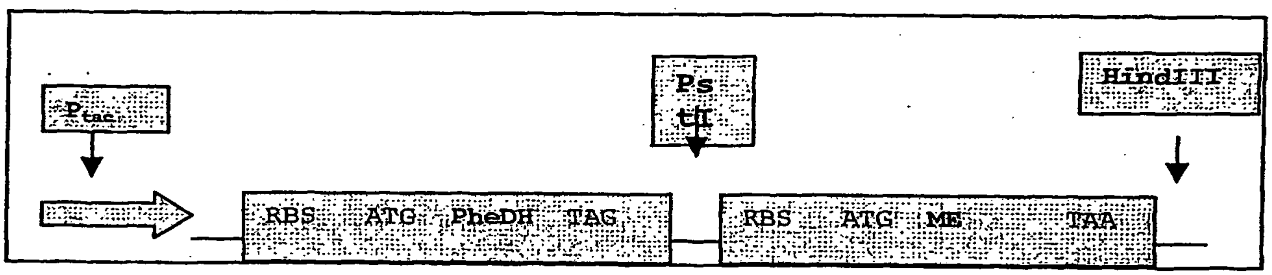
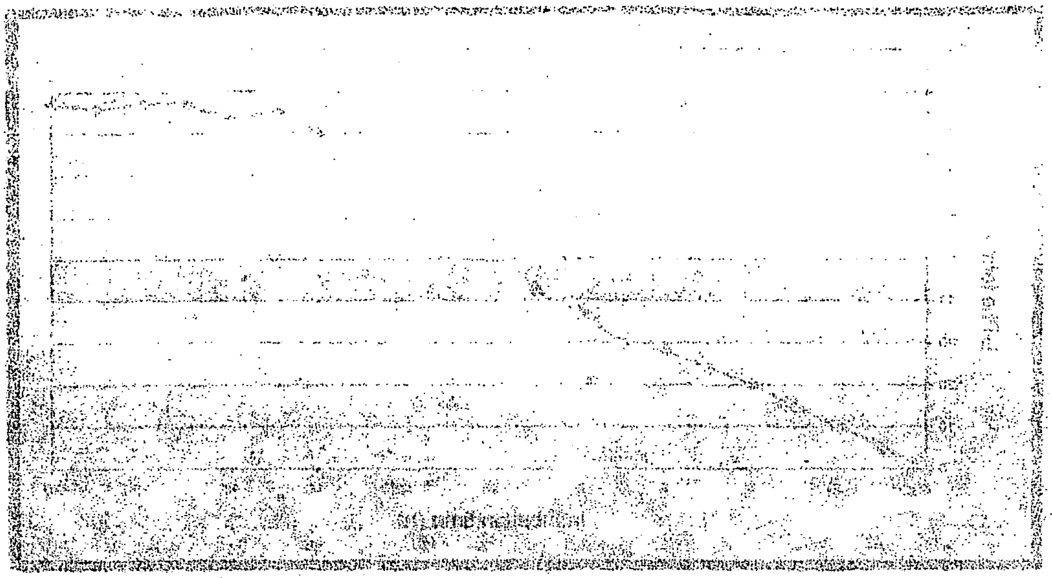

L-Phe formation (%)
Incubation time (h)

Fig. 8:


Ptac
Pst I
HindIII
RBS ATG PheDH TAG
RBS ATG ME TAA



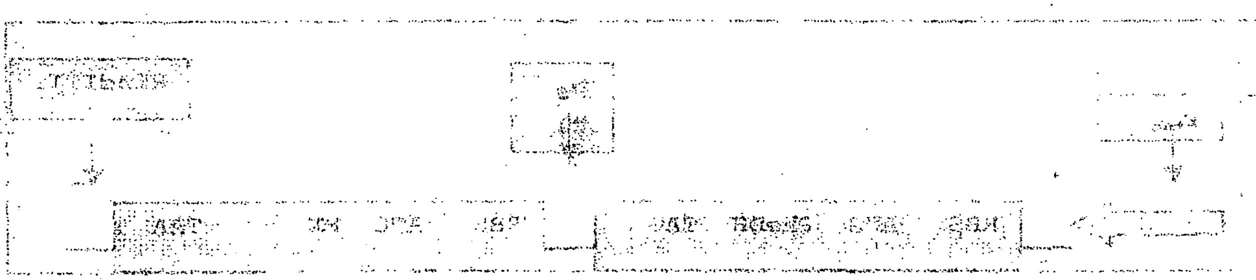


Fig. 9:

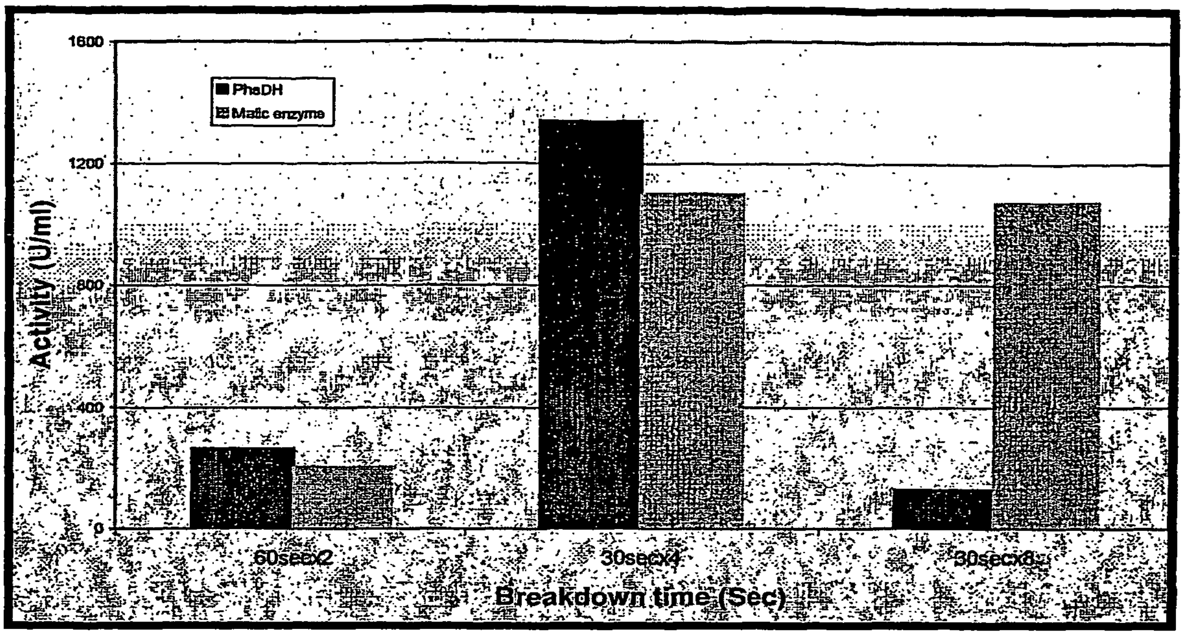
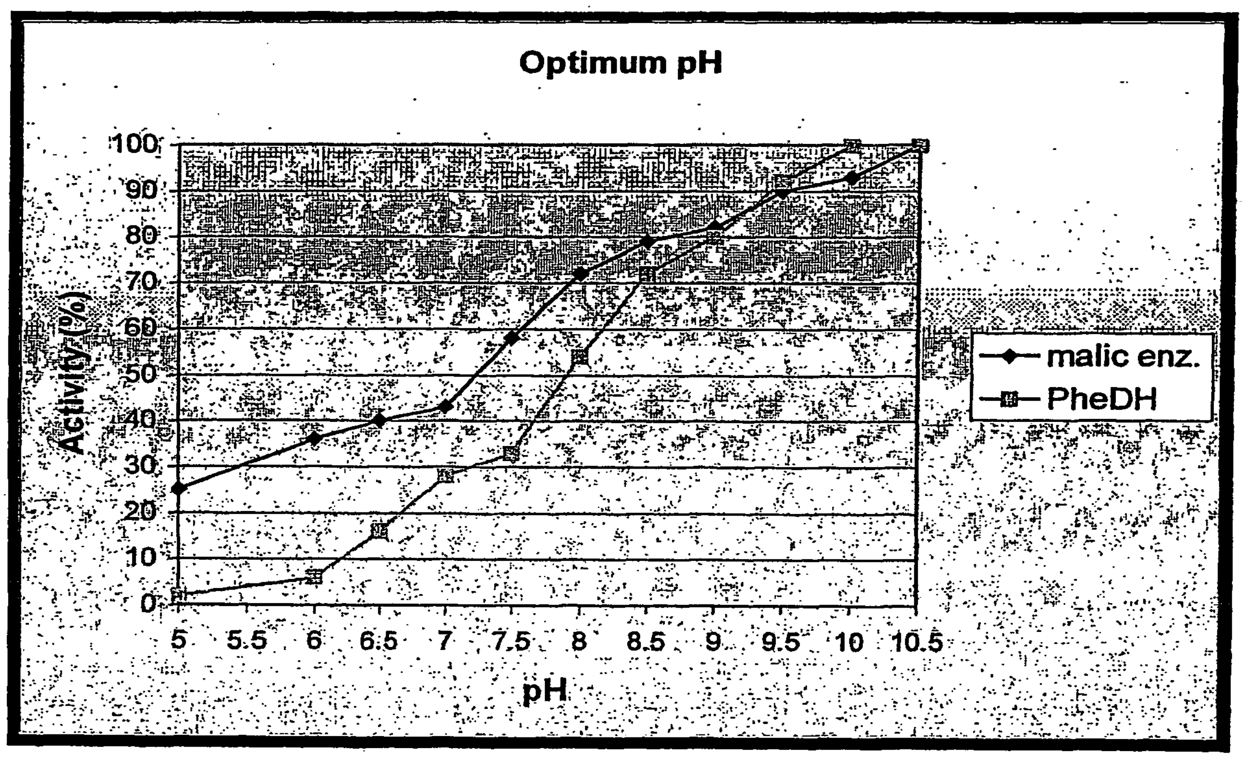
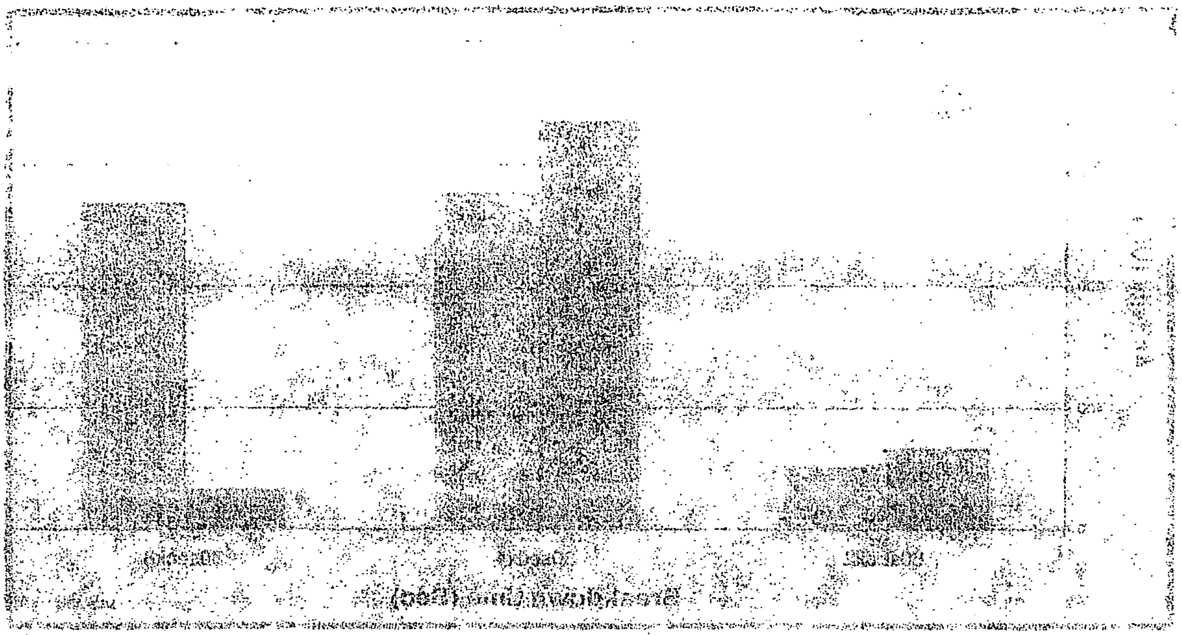

1600
1200
800
400
0
PheDH
Malic enzyme
Activity (U/ml)
60secx2
30secx4
30secx8
Breakdown time (Sec)

Fig. 10


Optimum pH
100
90
80
70
60
50
40
30
20
10
0
Activity (%)
5 5.5 6 6.5 7 7.5 8 8.5 9 9.5 10 10.5
pH
malic enz.
PheDH


Breakdown time (sec)

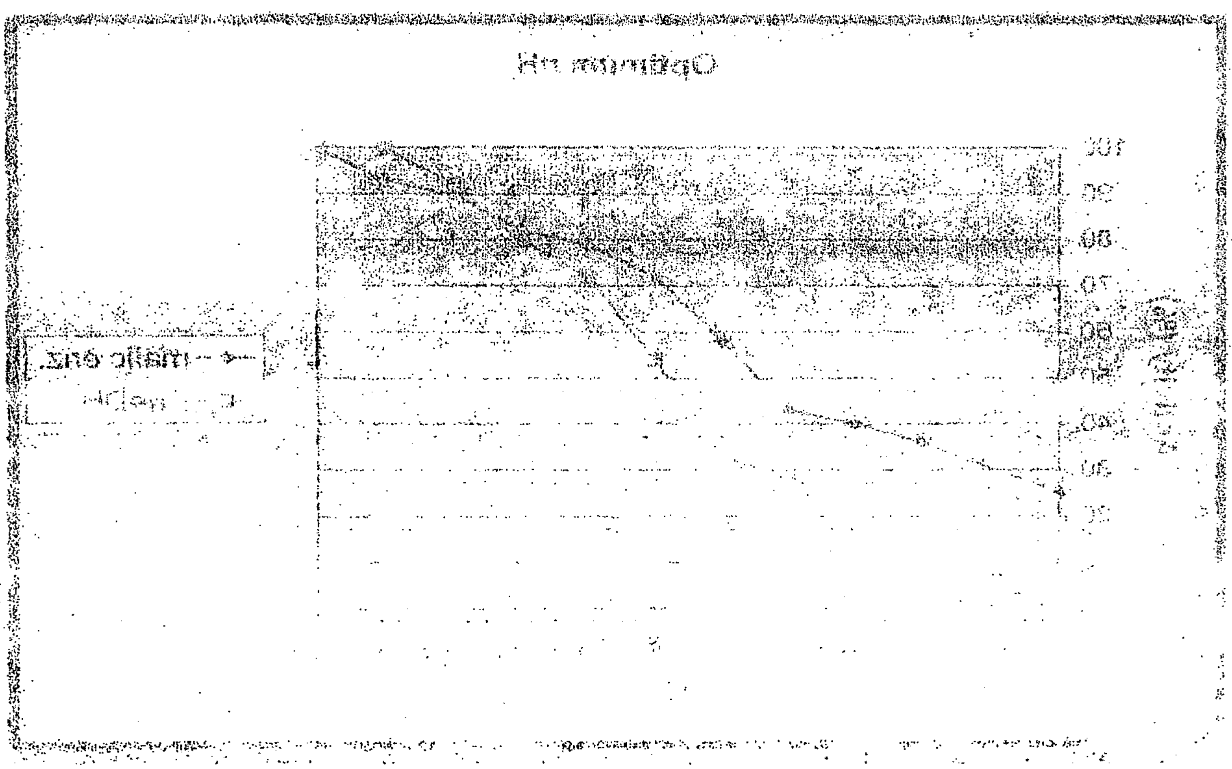

Optimum pH
Malic enz.

Fig. 11:

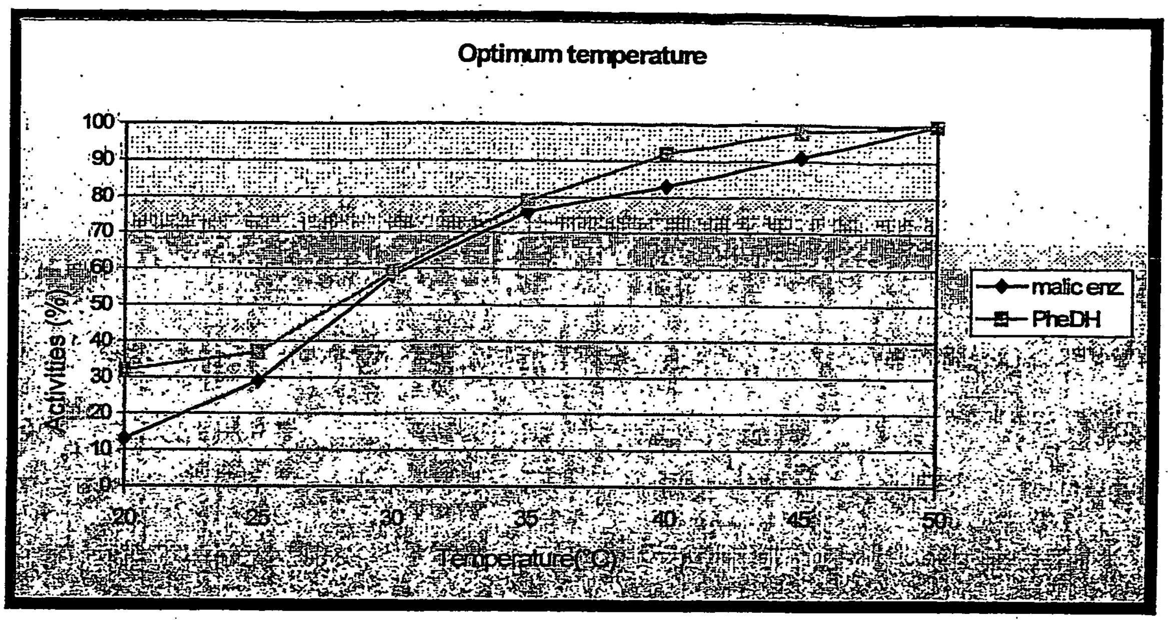
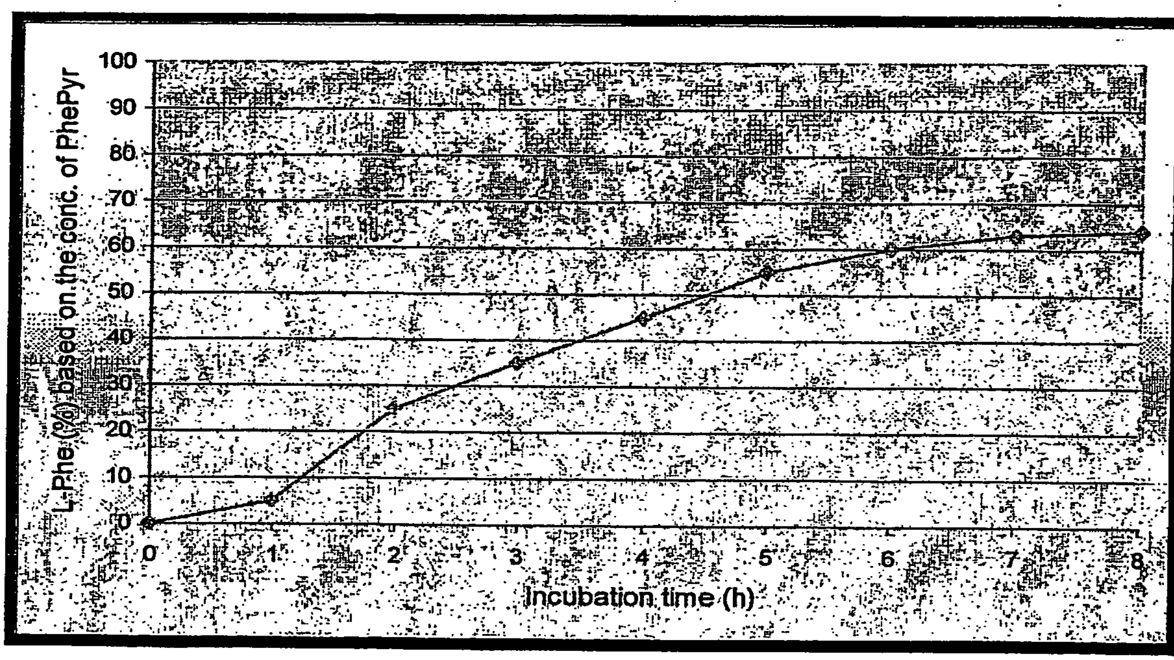

Optimum temperature
Activities (%)
100
90
80
70
60
50
40
30
20
10
0
20
25
30
35
40
45
50
Temperature(°C)
malic enz
PheDH

Fig. 12


L-Phe (%) based on the conc. of PhePyr
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
Incubation time (h)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/08631

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P13/04 C12P7/04 C12P41/00 C12P13/22 C12P13/06
C12N15/53 C12N9/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US 2002/042110 A1 (MITSUHASHI KAZUYA ET AL) 11 April 2002 (2002-04-11) paragraph '0162!	1-10
X	EP 0 385 415 A (MITSUBISHI PETROCHEMICAL CO) 5 September 1990 (1990-09-05) page 10; example 6	1-8

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 December 2003	18/03/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lopez García, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199001 Derwent Publications Ltd., London, GB; Class B05, AN 1990-003179 XP002264717 -& JP 01 285193 A (DAICEL CHEM IND LTD), 16 November 1989 (1989-11-16) page 156 abstract	1-8
X	DATABASE WPI Section Ch, Week 198630 Derwent Publications Ltd., London, GB; Class B05, AN 1986-194622 XP002264718 -& JP 61 128895 A (TAKARA SHUZO CO LTD), 16 June 1986 (1986-06-16) abstract	1-8
X	GU K F ET AL: "PRODUCTION OF ESSENTIAL L BRANCHED-CHAIN AMINO ACIDS IN BIOREACTORS CONTAINING ARTIFICIAL CELLS IMMOBILIZED MULTIENZYME SYSTEMS AND DEXTRAN-NAD" BIOTECHNOLOGY AND BIOENGINEERING, vol. 36, no. 3, 1990, pages 263-269, XP001156578 ISSN: 0006-3592 page 266; figures 2,7	1-8
X	SUYE S: "COENZYME REGENERATION WITH MALIC ENZYME REACTION SYSTEM" RECENT RESEARCH DEVELOPMENTS IN FERMENTATION AND BIOENGINEERING, XX, XX, vol. 1, no. 1, 1998, pages 55-64, XP000982890 figure 5; table 1	1-8
X	BOMMARIUS A S ET AL: "BIOCATALYSIS TO AMINO ACID-BASED CHIRAL PHARMACEUTICAL-EXAMPLES AND PERSPECTIVES" JOURNAL OF MOLECULAR CATALYSIS. B, ENZYMATIC, ELSEVIER, AMSTERDAM,, NL, vol. 5, 1998, pages 1-11, XP002929513 ISSN: 1381-1177 figure 5	1-8

INTERNATI[redacted]AL SEARCH REPORT

Inf[redacted]ion on patent family members

Int[redacted]pplication No
PCT/EP 03/08631

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			AU	2730399	A	27-09-1999
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			WO	9946363	A1	16-09-1999
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